In the Specification

On page 25, line 6, above "Detailed Description of the Invention" please insert the following paragraph:

--**Figure 4** is a diagram illustrating a regeneration scheme for cotton of this invention. The abbreviations G0 through G3, $MS_{zn\text{-}g,}$ 1/2 G0 and GRM_{gn} are described in Table 14 hereof.--

Please substitute the following paragraphs:

Page 98, line 21 through page 99, line 3:

Cotton was transformed essentially as disclosed by Firoozabody, E. *et al.* (1987) *Plant Mol. Biol.* 10:105-116, and Firoozabady, E. U.S. Patent Application Serial No. 07/076,339. The Firoozabady application reads, in pertinent part: Cotton (genus *Gossypium*) is an important commercial crop. Fiber-producing members of this genus are *G. arboreum*, *G. herbaceum*, *G. hirsutum*, *G. barbadense*, *G. lanceolatum*, all the foregoing being cultivated species, and *G. tomentosum*, *G. mustelinum* and *G. darwini* which are wild-type species. "Cotton," R.J. Kohel *et al.* eds. (1984), American Society of Agronomy, Inc., p. 52. In the United States, *G. hirsutum* is the major cultivated species. A number of different varieties are cultivated in different parts of the country, classified into Acala, Delta, Plains and Eastern. The Acala varieties grown in the Southwest are predominantly Acala 17's, and in California are the SJ series. Delta varieties include Stoneville and Deltapine. Plains varieties include Lankart and Paymaster, and Eastern varieties include Coker and McNair. Cotton, *supra*, p. 203-205. Southwestern varieties also include the GSA varieties.

Despite success in regenerating a number of plants such as tobacco and petunia, investigators have had substantial difficulties with regeneration of cotton (*Gossypium hirsutum L.*). Methods of regenerating this valuable crop plant from somatic tissue are desirable so as to enable transformation of cotton with foreign DNA conveying valuable agronomic traits. Limited success has been obtained in the regeneration of easily regenerable Class 1 genotypes of cotton such as the Coker varieties grown primarily in the eastern United States, but to date no methods have been available for the regeneration and transformation of Class 2 agronomic genotypes such as those of the Acala, Delta and Plains types which make up the important crop varieties of the remainder of the United States. The methods by which this invention achieves regeneration involve adjustments of auxin/cytokinin

ratios in somatic embryogenesis induction media. No prior disclosures teach or suggest such adjustments for the regeneration of any variety of cotton.

A practical, reproducible, efficient method for regenerating fiber-producing species of cotton as provided herein is useful in rapid multiplication of plants produced by conventional breeding methods and in genetic engineering of plants wherein foreign genes are introduced into plant cells and the cells are regenerated to form whole fertile plants.

Ammirato, P.V. (1983) "Embryogenesis," in Handbook of Plant Cell Culture, Evans, D.A., *et al.*, eds. *1*:82-123 provides a general discussion of somatic embryogenesis as a method of plant regeneration. This article at page 84 describes the basic somatic embryogenesis protocol as involving a primary medium with an auxin source and a second medium devoid of growth regulators. During the primary culture the tissue underwent differentiation to produce a mass of unorganized cells and cell clusters, and transfer to a second medium prompted either initiation of embryonic development, or as it was later thought, embryo maturation. At page 99, auxins or auxins in combination with cytokinins are said to be essential to the onset of growth and the induction of embryogenesis. At page 100, cytokinins are characterized as important in fostering somatic embryo maturation and cotyledon development. This article indicates that embryogenesis protocols have a high degree of specificity for the type of plant being regenerated. No reports of cotton regeneration are described in this article, nor is a callus initiation medium having a high cytokinin/auxin ratio followed by an embryo induction medium having a high auxin/cytokinin ratio as used in this invention suggested or disclosed.

The only prior report of cotton transformation producing whole plants known to applicant is disclosed by P. Umbeck, et al. (1987), in "Genetically Transformed Cotton (*Gossypium hirsutum L.*) Plants," Bio/Technology *5*:263-266, describing regeneration of Coker varieties 310, 312 and 5110 from hypocotyl sections transformed with a kanamycin resistance gene (*npt*II) and a chloramphenicol acetyltransferase (*cat*) gene. After incubation with *Agrobacteria*, the hypocotyl sections were placed on a medium containing equal amounts of auxin and cytokinin (0.1 µg/ml each of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylaminopurine (kinetin) as well as 5-50 µg/ml kanamycin sulfate. After tissue amplification, embryogenic tissues were transferred to a regeneration medium without phytohormones. Mature embryos (4 mm or more with cotyledon and radicle structures) were transferred to Stewart and Hsu medium (J. Stewart et al. (1977), "In-ovulo embryo culture and seedling development of cotton (*Gossypium hirsutum L.*)" Planta *137*:113-117) with indole acetic acid (IAA), 6-benzylaminopurine (BA) and gibberellic acid (GA) all at 0.1 µg/ml.

Tissue incubation was done at 30 degrees C. No regeneration of genotypes other than Coker was reported. This article characterizes the inability to regenerate plants as a major obstacle to practical application of transformation techniques. The discussion of this article does not constitute any representation that it constitutes prior art which may be properly applied against the claims hereof.

Prior reports of cotton regeneration include the following:

J. Stewart et al. (1977) *supra*, disclose the culture of zygotic embryos of *Gossypium hirsutum L.* cv. Hancock on the high-salt media BT and BTP (the latter containing phytohormones) of C.A. Beasley, et al. (1973) Amer. J. Bot. *60*:130 modified by the addition of ammonium ion. No regeneration from somatic tissue was reported.

The first report of regeneration of a domestic cotton variety was that of G. H. Davidonis, et al. (1983), "Plant Regeneration from Callus Tissue of *Gossypium hirsutum* L.," Plant Science Letters *32*:89-93, reporting the regeneration of plants from Coker 310 genotype callus derived from culture of seedling cotyledon. Callus initiation was done on LS medium (E.M. Linsmaier et al. (1965) Physiol. Plant., *18*:100) containing 2 mg/l α-naphthalene acetic acid (NAA) and 1 mg/l kinetin. After three months callus tissue was subcultured on modified LS medium containing 30 g/l glucose, 1 mg/l NAA and 0.5 mg/l kinetin. Over a three-year period a few pro-embryoids were formed. This article reports increased embryogenic potential of callus tissue after growth on media without hormones, and that embryoid growth was slower in media lacking hormones than containing NAA and kinetin. The three-year embryo induction period and low efficiency of embryo formation indicate that this article is describing an adventitious observation. This article does not provide a teaching or protocol enabling practical, usable somatic embryogenesis and regeneration of cotton and discusses only the Coker 310 genotype.

Prior difficulties in regeneration of cotton varieties other than Coker are illustrated by the disclosure of R. C. Shoemaker, et al. (1986), "Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.)," Plant Cell Reports *3*:178-181. This article reports evaluation of seventeen cultivars on three callus initiation and three callus maintenance media. Seedling hypocotyl sections were used. The best initiation medium was a medium containing MS salts with 2 mg/l indole-3-acetic acid (IAA), and 1 mg/l kinetin. The best maintenance medium was one containing MS salts with 10 mg/l N6-(isopentenyl)-adenine (2iP) and 1 mg/l NAA. This medium produced the most vigorous and healthy calli but was not embryogenic. The maintenance medium was found not to be necessary for induction of embryogenic callus, and callus was initiated directly on MS medium with 3% glucose and 2 mg/l NAA and 1 mg/l kinetin, then switched to the

same medium using 3% sucrose instead of 3% glucose for induction. Only Coker 201 and 315 varieties could be regenerated.

H. J. Price, et al. (1979), "Somatic Embryogenesis in Suspension Cultures of *Gossypium klotzchianum* Anderss," Planta *145*:305-307, purports to comprise the first report of reproducible somatic embryogenesis in a species of the genus *Gossypium*. The species, however, is a wild-type, nonfiber-producing species rather than a domestic cotton species. The explants used were seedling hypocotyls. The use of a "pre-culture" containing a high cytokinin concentration (2iP (N^6-(2-isopentyl)-adenine) at a concentration of 10 mg/l) was disclosed as useful prior to making a suspension culture of the callus in media containing 0.1 mg/l 2,4-D and 20 g/l sucrose, but no cytokinin. This "pre-culture" was done following culture on an MS medium containing 2.0 mg/l IAA, and 1.0 mg/l kinetin. It was found that it was essential to somatic embryo formation that after suspension culture, the cells be transferred to a B5 medium containing glutamine (a medium also containing 0.5 mg/l 2,4-D was used), and that when the "pre-culture" with high 2-iP was not used prior to the suspension culture somatic embryos did not form. The authors stated that further testing would be required to determine if other cytokinins than 2-iP or lower concentrations would be effective in "pre-cultures". This article does not disclose the regeneration of domestic varieties of cotton, nor does it disclose a protocol involving a high cytokinin/auxin callus initiation medium followed by a high auxin/cytokinin embryo induction medium.

J. J. Finer et al. (1984), "Initiation of callus and somatic embryos from explants of mature cotton (*Gossypium klotzchianum* Anderss," Plant Cell Reports *3*:41-43 describes unsuccessful attempts to regenerate plants from embryos produced from stem and petiole sections of the above wild species. High 2iP media were used followed by suspension culture in a medium containing glutamine and 2,4-D in which embryos were induced. Embryo development took place in auxin-free media. Embryos were abnormal, and efficiencies were low.

R. H. Smith, et al. (1977), "Defined Conditions for the Initiation and Growth of Cotton Callus in Vitro In *Gossypium arboreum*," In Vitro *13*:329-334 describes nutrient media useful for callus proliferation and subsequent growth of subcultures. Seedling hypocotyl explants were found superior to cotyledon or leaf explants. MS media containing IAA (2 mg/l) and kinetin (1 mg/l) were found best of the combinations of auxins and cytokinins tested for callus proliferation, and media containing 2 mg/l NAA and 0.5 mg/l BA, or 1 mg/l NAA and 5-10 mg/l 2iP were found to be best for subculture. The authors report one adventitious case of plantlet regeneration (no further details available) which they did not pursue. This disclosure was not directed to somatic embryo production.

Reproducible regeneration protocols with respect to fiber-producing cotton species have thus been limited to Coker varieties of *G. hirsutum*.

T. L. Reynolds (1986), "Somatic Embryogenesis and Organogenesis from Callus Cultures of *Solanum Carolinense*," Amer. J. Bot. 73:914-918 describes culture of stem segments of a species of horse-nettle on a medium supplemented with 10 mg/l 2,4-D and 1 mg/l kinetin for callus initiation, with subculture on a medium lacking 2,4-D but containing a cytokinin for embryo production and regeneration. These protocols directly teach against the protocols used by applicant herein involving a callus initiation medium having a high cytokinin/auxin ratio and an embryo induction medium having a high auxin/cytokinin ratio.

Applicant co-authored a poster displayed at a conference at the University of California at Davis, California August 24-29, 1986 on "Tailoring Genes for Crop Improvement" entitled "Transformation and Regeneration of Cotton, *Gossypium hirsutum* L." This poster described transformation and regeneration of cotton.

In a nonenabling abstract for the Thirty-eighth Annual Meeting of the Tissue Culture Association held May 27-30, 1987, entitled "Transformation of Cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and Regeneration of Transgenic Plants," applicant and others report transformation and regeneration of cotton. An oral presentation on the subject was made by applicant.

Zhou, G.-Y. *et al.* (1983), "Introduction of Exogenous DNA into Cotton Embryos," Meth. Enzymol. *101*:433-481 discloses a method for injection of DNA from *G. barbadense* into *G. hirsutum* ovaries. Mutations were observed in progeny, however, this method does not allow for the transformation of plants with selected foreign genes governing particular desired traits as do the methods of this invention.

Figure 4 is a diagram illustrating a regeneration scheme for cotton of this invention. The abbreviations GO through G3' MS_{zn-g}, $1/2G_0$ and GRM_{gn} are described in Table 14 hereof.

A process is provided for regenerating a whole plant of the genus *Gossypium*. Preferably the plant is of a fiber-producing species selected from the group consisting of *G. arboreum*, *G. herbaceum*, *G. hirsutum*, *G. barbadense*, *G. lanceolatum*, G. *tomentosum*, *G. mustelinum*, and *G. darwini*. More preferably, the species is a cultivated fiber-producing species selected from the group consisting of the first five above-named species. Of these, *G. hirsutum* is the most useful for cultivation in the United States and regeneration of this species is a preferred embodiment of this invention.

As discussed above, the Coker varieties in general, and in particular, Cokers 310, 312, 5110, 201, and 315 are regenerable by means of prior art techniques, i.e. the use of a primary medium for callus initiation containing low concentrations of auxins (.1 mg/l to 10 mg/l and equal or lower concentrations of cytokinin, and a secondary medium containing the same hormones or no hormones for embryo induction or maturation). Other varieties of *G. hirsutum* were not responsive to these techniques. Easily regenerable varieties such as Coker are termed "Class 1 varieties" herein. A "Class 1 variety" is one regenerable by means of a basic somatic embryogenesis protocol as described by Ammirato, P. V., *supra* involving a primary medium with an auxin source and a secondary medium devoid of growth regulator or having cytokinins. Class 1 varieties respond to the callus initiation, embryogenic callus induction and embryogenic callus formation steps of the protocol of this invention much sooner than other, more hard to regenerate cotton varieties which are designated as "Class 2 varieties". Class 2 varieties are not regenerable by prior art methods and respond more slowly to the protocol of this invention than Class 1 varieties, all as described below.

Tissue from the plant to be regenerated is first placed on a callus initiation medium. Tissue explants useful in practicing this invention include hypocotyl, cotyledon and leaf sections, preferably taken from precociously germinated seedlings. Hypocotyl segments were especially useful in obtaining regeneration of Class 2 varieties; however, cotyledon sections are also useful. Immature embryos, or portions thereof may also be used. Immature embryos are fully developed but not yet hardened.

The preferred embodiment of this invention involves the use of a callus initiation medium having a high cytokinin to auxin ratio to proliferate callus. A number of cytokinins are well known to the art, and more fully described below. The most preferred cytokinin of this invention is 2iP. Auxins are well known to the skilled worker and described in the prior art as well. The preferred auxin for use in this invention is NAA. A high cytokinin auxin ratio is defined herein to be greater than about 10:1; preferably the ratio is at least about 30:1 to 50:1 and can be as high as 100:1. The cytokinin concentration may be as high as about 10 mg/l, but not less than about 1 mg/l, and the auxin concentration should not be more than about 1 mg/l and may be as low as about 0.01 mg/l.

The callus initiation culture is continued until the callus has proliferated to about 5-10 times its original size, and until the calli are sufficiently mature that the auxins will not be toxic to the cells, generally about two to three weeks for the Class 1 varieties and about four to five weeks for the Class 2 varieties.

After the callus initiation step, the callus is transferred to an embryogenic callus induction medium having high auxin to cytokinin ratio. A "high" ratio of auxin to cytokinin for this purpose is defined as being at least about 1:1. Preferably this ratio is at least about 3:1 and more preferably about 50:1 to about 100:1. The auxin concentration should not be more than about 5 mg/l or less than about 1 mg/l, and the cytokinin concentration should not be more than about 1 mg/l and can be as low as 0.0 mg/l. The tissues are maintained on this medium until embryogenic callus is induced, characterized by production of proembryoids having globular and heart-shaped structures --generally about two to three weeks for Class 1 varieties and about five to six months for Class 2 varieties.

The calli are then transferred to suitable media for embryogenic callus formation, embryo maturation, embryo germination, and plant regeneration.

In a preferred embodiment of this invention utilizing the above preferred protocol, the regenerated plant is a Class 2 cultivar.

Also in a preferred embodiment of this invention, following the embryogenic callus induction stage, the calli are transferred to an embryogenic callus formation medium without phytohormones for maximum embryogenic callus production.

Culturing on the hormone-free medium is preferably continued until embryo maturation occurs, i.e. somatic embryos have a pair of cotyledons, green color and are about three to about 12 mm in size. The calli can be maintained on this medium for as long as desired. Preferably, however, after about three to four weeks for Class 1 varieties and about six to seven weeks for Class 2 varieties, the calli are transferred to a new medium for another two to three weeks. This new medium is preferably hormone-free medium, but optionally, may be medium containing small amounts of auxin, preferably NAA at about 0.1 mg/l, a cytokinin, preferably zeatin at about 1 mg/l, and reduced carbohydrate concentration, preferably about 1.5% glucose. The preferred hormonal medium for this purpose is $MS_{zn\text{-}g}$ as shown in Table 14.

Embryo germination media for use following the embryo maturation step described above are known to the art. A preferred embryo germination medium is a low ionic strength medium such as Stewart and Hsu medium without the added ammonium. Preferably this medium is supplemented with a gibberellin and an auxin, preferably about 0.1 mg/l gibberellic acid and about 0.01 mg/l NAA, and preferably glucose (0.5%) is used instead of sucrose.

As shown in Figure 4, in a preferred embodiment hereof, after about two to three weeks on the preferred embryo germination and plantlet development medium, GRM_gn, the plantlet is transferred to a plant development medium such as a phytohormone-free medium containing reduced

salts and carbohydrate (preferably about 1/2 MS salts and about 1.5% glucose) or culture may be renewed on a germination medium, preferably GRM_{gn}.

Growth to a whole fertile plant is continued under greenhouse conditions.

The regeneration protocol provided herein is particularly valuable for use in genetic engineering to produce whole, fertile transformed cotton plants. Transformation (incorporation of foreign DNA into the plant genome) may be accomplished by any means known to the art, preferably by infection with *Agrobacterium tumefaciens* containing the desired foreign DNA. The transformed tissues are cultured as above for regeneration into whole plants. "Foreign" refers to any DNA or genes which do not occur naturally at their new location in the host plant's genome. Foreign genes may be genes with their own promoters or chimeric genes derived from *Gossypium* or other organisms. Preferably, the foreign DNA or genes confer an identifiable phenotype on the regenerated host plant and/ or its progeny by which the plant is distinguishable from naturally occurring plants. Such phenotypes conferred by foreign DNA include performance on laboratory tests such as Southern, northern and western blot procedures. Also preferably the transformation method allows for the insertion of selected genes or DNA into the cotton genome. A "selected gene" is a gene governing a particular trait which it is desired to confer on the recipient plant (as distinguished from unselected genes which may be transferred by methods such as injection of DNA extracted from other organisms and containing an unknown type and quantity of genes). Other types of "selected" foreign DNA might be particular isolated promoters or enhancers transferred to the recipient genome to perform their known functions.

By means of this invention, whole transformed cotton plants, preferably Class 2 varieties, are obtained which can express the foreign DNA or genes contained therein, e.g. foreign promoters and enhancers can be expressed to operate to turn on or enhance the activities of other genes, and foreign genes can be expressed to produce RNA and protein.

By means of this invention a regenerated plant preferably of a Class 2 *Gossypium* genotype transformed to contain foreign DNA and having a phenotype conferred by said foreign DNA by which said plant can be distinguished from a natural occurring plant is produced. Progeny of these plants may also be produced, as well as seeds of said plants and progeny plants. Any plant produced by the methods of this invention which is not phenotypically distinguishable from a naturally-occurring plant, is nevertheless considered to be within the scope of equivalents of plants claimed herein which are phenotypically distinguishable.

This invention has provided an important improvement in methods for regenerating cotton plants, which methods involve culturing somatic tissue of said plants on suitable media to cause callus formation and whole plant regeneration, the improvement of this invention comprising using somatic tissue of a Class 2 genotype of a *Gossypium* species.

COTTON REGENERATION EXAMPLE

Tissue from a plant of genus *Gossypium*, preferably a fiber-producing species thereof, and more preferably, a species of *G. hirsutum*, is regenerated to produce a whole plant. In the preferred embodiment hereof, the genotype used is a Class 2, difficult-to-regenerate genotype, preferably a genotype of *G. hirsutum*, and more preferably a GSA genotype.

The tissue is preferably obtained from seedlings about seven to about 10 days old, and preferably the seedlings are grown from immature seeds taken from cotton bolls which are about three to about five centimeters in diameter, about 40 to about 60 days after pollination. Incubation procedures for growing up seedlings are known to the art, for example as described in Firoozabady, E., *et al.* (1986), IC "Isolation, Culture, and Cell Division in Cotyledon Protoplasts of Cotton (*Gossypium hirsutum* and *G. barbadense*)," Plant Cell Rep. *5*:127-131. The medium used for production of seedlings is preferably a G_0 medium not containing hormones as described in the Cotton Regeneration and Transformation Examples hereof.

The explants used for tissue culture are preferably cotyledon pieces, preferably approximately 0.6 cm^2 in surface area, or hypocotyl sections, preferably about 5-8 mm in length, or leaf pieces approximately 0.6 cm^2 in surface area, taken from seedlings. These explants are taken when the seedlings are large enough to provide tissue of sufficient size, and preferably the seedlings are approximately three weeks old when the explants are taken.

Seedlings may be grown from immature seeds or germinated by culturing dry seeds. Tissue from immature (fully developed but not hardened) embryos may also be used.

The initial culture medium (callus initiation medium)is a medium containing a high cytokinin/auxin ratio. The cytokinin concentration must not be so high that it is toxic to plant cells, but must be sufficiently high that it stimulates growth of plant cells, and the auxin concentration must not be so high that it is toxic, but must be sufficiently high that it induces cell proliferation. Preferably the cytokinin concentration is between about 1.0 and about 10.0 mg/l, and more preferably between about 3 and about 5 mg/l. The auxin concentration is preferably between about 1 and about 0.01 mg/l, and more preferably between about 0.1 and about 0.2 mg/l. The cytokinin to auxin ratio is

preferably between about 10:1 and about 100:1, and more preferably between about 30:1 and about 50:1. The most preferred callus initiation medium is the G_2 medium described in Table 14 containing 5 mg/l 2iP and 0.1 mg/l NAA along with MS salts (Gibco, Grand Island, New York), and glucose at 3.0% (w/v), along with 100 mg/l myo-inositol, 0.4 mg/l thiamine HC1, 0.2% Gel-rite (Kelco, San Diego, California), at pH 5.9. As will be understood by those skilled in the art, other basal media and carbohydrate sources may be substituted for those specifically described herein. Other basal media known to the art as useful for regeneration are SL, V5 and L2. Preferably the auxin is from the NAA family, defined herein to include IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), and NAA (alpha-naphthaleneacetic acid). Other auxins known to the art are 2,4-D and related auxins of the 2,4-D family, defined herein to include 2,4-D (2,4-dichlorophenoxyacetic acid), Picloram (4,amino-3,5-6-trichloropicolinic acid), pCPA (parachlorophenoxyacetic acid), 2,4,5-T(2,4,5-trichlorophenoxyacetic acid), and Dicamba (2-methoxy,3-6-dichloro-o-anisic acid). Many cytokinins are known to the art. Examples of useful cytokinins are ADE (adenine sulfate), kinetin (6-furfurylaminopurine), BA (6-benzylaminopurine), and zeatin. Concentrations of 2,4-D higher than about 0.1 mg/l, however, have been found to be toxic to cotton cells, and 2,4-D is thus not a preferred auxin.

This initial high cytokinin culture is essential to achieving the efficiency of embryo induction in later culture stages required for a practical cotton regeneration method.

The callus produced on the callus initiation medium should be midfriable, rather than extremely hard (nonfriable) or extremely friable, as midfriable callus gives the best production of embryogenic callus in subsequent culture steps.

For callus initiation, a high temperature, preferably around 30°C is preferred, but is not critical. Light intensity during this phase of regeneration does not appear to be critical, and may vary from complete darkness up to high intensities, such as 90 $\mu E/m^2/s$.

After about two to about three weeks of callus initiation for Class 1 varieties (e.g. Cokers), or about seven to about nine weeks for Class 2 varieties (e.g. GSA'S), the calli are transferred to an embryogenic callus induction medium. This step is essential for regeneration of Class 2 varieties but may be omitted for Class 1 varieties. This medium contains a high auxin/cytokinin ratio. The auxin concentration should be high enough so that it stimulates the process of embryogenesis, but not so high that it is toxic to plant cells; preferably the auxin concentration is between about 1 and about 5 mg/l, and more preferably between about 3 and about 5 mg/l. The cytokinin concentration should be high enough so that it induces embryogenesis, but not so high that it prevents embryogenesis.

Preferably the cytokinin concentration is between about 0 and about 1 mg/l and more preferably between about 0.05 and about 0.1 mg/l. In the preferred embodiment described in the Example, media containing NAA at 5 mg/l and 2iP at either 1 or 0.1 mg/l, and also containing the additional components described above in connection with the G_2 medium are used. These two media are respectively G_1 and G_3. Again, as will be understood by those skilled in the art, equivalent components may be used, but it is necessary to maintain a high auxin/cytokinin ratio in this medium. Glucose is the preferred carbohydrate for use in the embryogenic callus induction medium, as well as for all the culture media used in this invention, as sucrose promotes production of phenolics and calli cultured on this medium have been observed to turn brown, and eventually to die. G_3, described in Table 14, is the preferred embryogenic callus induction medium.

Embryogenic callus formation may be observed on the embryogenic callus induction medium when the culture is kept on the medium long enough to exhaust the hormones. Preferably, however, the callus is transferred to hormone-free medium for embryogenic callus formation.

In the preferred embodiment after about three to four weeks on the embryogenic induction medium for the Class 1 genotypes, or about four to five months for the Class 2 genotypes, the callus is transferred to a phytohormone-free medium for embryogenic callus formation. Stages of somatic embryo development are well defined in the art, e.g. by Shoemaker, R. C., *et al.* (1986), *supra*, and Finer, J. J., *et al.* (1984) , *supra*. Immature somatic embryos lack well defined organs, whereas normal, mature somatic embryos are those with a pair of cotyledons and normal morphology (i.e., green color and about three to about 12 mm in size). The hormone-free medium used for embryogenic callus formation is preferably the medium used for callus initiation or for embryogenic callus induction without the phytohormones, preferably G_1, G_2 or G_3 medium without the hormones. As will be apparent to those skilled in the art, however, other hormone-free nutrient medium may be used.

Embryogenic calli are recovered at a frequency of about 10% to about 85% depending upon the genotype used, the Class 1 genotypes producing calli faster and at higher frequencies. Embryogenic calli are characterized as midfriable, creamy, granular tissues. They are generally observed first as sections of calli or sometimes whole brown, midfriable calli become embryogenic. This embryogenic callus contains proembryoids (globular and heart-shaped structures).

For embryogenic callus formation, high temperature (e.g. around 30°C), and low light, for example, about 9 $\mu Em^2/s$, are preferred.

The embryogenic callus after about two to three weeks on the embryogenic callus formation medium for a Class 1 variety or about four to six weeks for the Class 2 varieties, is subcultured on the same medium, or on a medium containing a cytokinin, preferably zeatin, an auxin, preferably NAA, and reduced carbohydrate. Preferably the auxin is present at between about 0 and about 0.2 mg/l, more preferably between about 0.01 and about 0.1 mg/l, and preferably the cytokinin is present at between about 0.5 and about 3.0 mg/l, and more preferably at a concentration between about 0.5 and about 1 mg/l. The medium MS_{zn-g} as described in the Examples hereof is the most preferred of such supplemented media. This medium contains 0.1 mg/l NAA and 1 mg/l zeatin together with MS salts, 1.5% (w/v) glucose, and the other nonhormone components of the G1, G2 and G3 media. For this final embryo maturation step, however, the nonsupplemented medium G_0 is most preferred. The use of 2,4-D or pCPA as the auxin in the supplemented medium, or reduction of the MS salts to one-half strength in either medium reduced the capacity of the medium for embryo production and maturation.

Mature embryos vary in size from about 2 to about 12 mm, have between about one and about four cotyledons, have variable hypocotyl lengths and vary in color from pale yellow to light green to dark green and occasionally albino most likely due to tissue culture art (but this albinism is not a heritable character).

After about two to three weeks on the embryo maturation medium for both Class 1 and Class 2 genotypes, mature embryos are transferred to an embryo germination and plant development medium. Germination media are known to the art, for example as described by Stewart, J. M. *et al.* (1977), *supra*. The germination medium may be modified by the addition of gibberellic acid (GA_3) at a concentration of between about 0 and about 1.0 mg/l, and preferably at a concentration of between about 0.05 and about 0.2 mg/l. The germination medium may also be modified to contain an auxin, preferably NAA, at a concentration of between about 0.0 and about 0.1 mg/l, and preferably not more than about 0.05 mg/l. Also preferably the medium uses glucose rather than sucrose, preferably at a concentration of between about 0.2 and about 1.5% (w/v), and more preferably at a concentration at between about 0.5 and about 1.0%. The most preferred medium is the medium described herein as GRMgn, containing about 0.1 mg/l gibberellic acid, about 0.01 mg/l NAA and about 0.5% glucose. The preferred medium is a low ionic strength medium. Ionic strength equal to the use of MS salts at a concentration of about 1 X MS salts caused burning and senescence of the tissues and caused problems in balancing root and shoot formation. The preferred medium will allow germination of at least about half of the mature somatic embryos. Iron at a concentration of between about 10 μm and

about 25 μm is an important component of the germination medium. Embryo germination or "conversion" is defined as the development of the apical area of the somatic embryo resulting in shoot production (true leaves).

Embryos developed well on the GRM_gn medium, as they do not tolerate higher salt concentrations well. If desired, a compound such as asparagine or ancymidol may be added to the GRM_{gn} medium. Plantlets grown on media containing these compounds have a dark green color and good root systems. These compounds may be added in concentrations which will be apparent to the skilled worker, preferably about 5 ppm ancymidol or about 100 mg/l asparagine.

High light intensity, (e.g. about 90 $\mu E/m^2/s$) are helpful for germination and plantlet development. During the embryo germination and plantlet development phase, it is also preferable to initially incubate mature embryos at high temperatures, e.g. about 30°C, for a few days to produce rapid germination, then lower the temperature to normal, about 25°C, for plantlet development.

After about one to two weeks on the embryonic germination and plantlet development medium, the plantlets, defined herein as germinated embryos having roots as well as shoots, may be transferred to media known to the art for further plant development. Preferably the plantlets and germinated embryos are transferred to Magenta cubes, such as GA7 Magenta cubes (Magenta Corporation, Chicago, Illinois), containing a phytohormone-free medium with reduced salts and carbohydrates, preferably about one-half MS salts and about 1.5% glucose. When plants are partially developed, e.g., about 8 to about 10 cm tall, having about 4 to about 6 leaves, they may be transferred to soil for maturation. Preferably the plants in soil are grown under initial high relative humidity for a gradual hardening off, and then under normal greenhouse conditions.

The foregoing procedure allows regeneration of full plants within about 14 to about 16 weeks for Class 1 varieties and about 8 to about 10 months for Class 2 varieties.

A well-defined, reproducible, and highly efficient plant regeneration scheme such as that defined above, is a prerequisite for transformation of cotton. Transformation may be performed by means known to the art for introduction of foreign DNA into plant cells and tissues. Once transformation of cells and/or tissues has been done, the transformed cells and tissues can be regenerated according to the above-described methods.

A preferred method of transformation involves the use of *Agrobacteria* to introduce foreign DNA into plant cells via infection. Means for inserting foreign DNA for transfer to plant tissues into *Agrobacteria* are well known to the art, and many vectors carrying such genes are known and readily available to the skilled worker. In a preferred embodiment, a coding region for the insecticidal

crystal protein (Bt) from *Bacillus thuringiensis* is used. Vectors containing Bt are described, for example, in U.S. Patent Application Number 848,733, incorporated herein by reference, and in Patent Application Number 617,321, also incorporated herein by reference.

The vector preferably contains a "marker" gene such as the neomycinphosphotransferase II (*npt* II) gene conferring kanamycin resistance. Other suitable markers are known to the art.

The *Agrobacteria* containing the vector to be used to insert foreign DNA into the plant tissue is cultured by means known to the art, preferably by growing on appropriate media such as agar media containing selection agents corresponding to the marker genes present on the vector, e.g., streptomycin and chloramphenicol. Other media, such as YEP medium, may also be used.

The *Agrobacterium* colonies are scraped off the selection medium and suspended in an appropriate liquid medium, such as YEP broth or minimal medium. Preferably, however, the bacteria are suspended in a liquid medium for cotton callus culture, preferably a callus initiation medium such as G_2 medium as described above, preferably used at a concentration of about 2-4 x 10^8/ml. The tissues are submerged in the bacterial suspension to assure adequate contact of the tissue to be transformed with the *Agrobacteria*. Concentrations are preferably less than 10^9 or 10^{10}, as such high concentrations tend to kill the tissue.

The tissue to be transformed may be any regenerable cotton tissue. Preferably cotyledon, hypocotyl and leaf sections from seedlings developed from immature embryos or germinated seedlings as described above are used. The tissue pieces may be of any manipulable size, and preferably are about 1/2 cm^2.

The tissue pieces are contacted with the *Agrobacterium* suspension, preferably by dipping in the liquid culture medium and shaking to ensure contact of all edges with the culture. To minimize *Agrobacterium* on the tissue, blotting dry, preferably with filter paper, is recommended. This reduces bacterial overgrowth on the plant tissues.

The tissue are co-cultivated with the *Agrobacterium*, preferably at about 25°C, and preferably under low light (10 $\mu E/m^2/s$), on a suitable medium, preferably a callus initiation medium such as G2 for long enough to ensure infection, preferably about two to three days. Preferably the tissue is plated on filter paper placed on the medium for co-cultivation to reduce bacterial overgrowth.

The infected tissues after co-cultivation are then placed on a medium to kill the *Agrobacteria*, for example by containing antibiotics known to the art. Examples of such antibiotics include carbenicillin, cloxacillin, and cefoxitin and preferably about 500 mg/l carbenicillin is used. The medium should also contain a selection agent to select for transformed tissue, corresponding to the

marker gene present in the vector. The selection agent should be present at a concentration high enough that untransformed cells do not grow, but not so high as to kill the transformed tissue. The selection agent used in the Examples is kanamycin sulfate, at a concentration of between about 15 and about 40 mg/l, preferably about 25 mg/l. Concentrations above about 50 mg/l, especially in the 100 to 150 mg/l range nonselectively kill the cells. Other selection agents known to the art, such as G418, hygromycin, bleomycin, and methotrexate are also useful. It is important that all cells have contact with the medium so as to ensure selection.

After a sufficient period of time to ensure selection and initiation of transformed microcalli, generally about one to about three weeks, the tissue is regenerated as described above. Preferably when microcallus grows to about 3 to about 4 mm, it is excised from the original explant and transferred to fresh medium. The time required for regeneration of transgenic plants, that is, the plants that contain the foreign DNA, is longer than for nontransferred plants due to inoculation of tissues with *Agrobacteria*, the selection pressures and effects of antibiotics.

All regenerable cotton varieties as described above may be transformed by the above methods, and by other methods known to the art, for example as described in S. H. Mantell, *et al.* (1985), Principles of Biotechnology, particularly chapter 4 thereof and the references referred to therein.

Dry seeds of *G. hirsutum* cultivars (Coker 201, 310, 315 and 4360; GSA 71, 75 and 78; GSC 25 (GSA and GSC are cultivars developed by GroAgri Seed Company (a subsidiary of Agrigenetics Corporation), Lubbock, Texas); G8160 (a breeding line from US Cotton Research Station, Shafter, California); GSA-Acala hybrids No. 21 and No. 22 (No. 21 is Acala SJ-C1 X GSC 20 and No. 22 is Acala SJ-Cl X GSA 74-7, 127 hybrid); and Acala SJ-2) were surface sterilized as described (Firoozabady, E. and DeBoer, D.L. (1986) Plant Cell Reports *5*:127-131, except that seeds were exposed to bleach for only 8-10 minutes, and germinated on G_O medium. Composition of media used in cotton regeneration is presented in Table 14 unless published elsewhere or modifications are mentioned in the text. Immature seeds were obtained from cotton bolls (3-5 cm diam., 40-60 days after pollination) of Coker 201 and 315 and GSA cultivars. Bolls were surface sterilized 20 minutes in 33% commercial bleach and rinsed twice in sterile distilled water. Immature seeds were manually delinted, seed coats were
removed, and the embryos (7-10 mm) were germinated on G_O medium and incubated as described in Firoozabady and DeBoer, (1986), *supra*.

Cotyledon pieces (approximately 0.6 cm^2 surface area) and hypocotyl sections (5-8 mm length) of 7- to 10-day-old seedlings and leaf pieces (approximately 0.6 cm^2 surface area) of 21-day-old plants were placed on callus initiation medium, G_2. Only hypocotyl sections were cultured from precociously germinated seedlings. After two to three weeks for Cokers or seven to nine weeks for GSA's, calli were transferred onto embryogenic callus induction media (G_1' G_3). Embryogenic calli were transferred to G_0 or MS_{zn-g} medium to produce mature somatic embryos. These were germinated on the medium suggested by Stewart and Hsu (1977), *supra*, modified by addition of 0.1 mg/l GA_3 (filter-sterilized), 0.01 mg/l NAA and 0.5% glucose instead of sucrose (GRM_gn). After one to two weeks, the germinated embryos were transferred to $1/2G_0$ or GRM_gn in GA7 Magenta cubes (Magenta Corp., Chicago, IL) to develop further. The plants (10-12 cm tall, 5-10 leaves) were transferred to soil under high relative humidity, gradually hardened off, and transferred to normal greenhouse conditions.

To study the effects of temperature and light intensity on different stages of tissue culture, tissues were incubated at 25±1°C or 30±1°C under high light intensity (90 $\mu E/m^2/s$) or low light intensity (9 $\mu E/m^2/s$) provided with cool white fluorescent lamps (GTE, Salem, MA) . Also, the effects on embryo quality and germination of different media containing additives such as ancymidol (Elanco Products, Co., Indianapolis, IN), asparagine, or different glucose concentrations were examined.

Immature somatic embryos were characterized by lack of well-defined organs. Normal somatic embryos are those with a pair of cotyledons and normal morphology (i.e., green color and 3-12 mm in size).

Results were as follows:

Callus initiation and proliferation. A range of media were tested for callus initiation from several cultivars. Medium G_2 (5 mg/l 2iP and 0.1 mg/l NAA, Table 14) was best for callus initiation and growth in many cultivars tested. Medium G2 was superior to G1, G3, EF18 (2 mg/l NAA and 1 mg/l kinetin, Shoemaker, R. C. *et al.* (1986) *supra.*), and the medium containing 2 mg/l IAA and 1 mg/l kinetin used by Smith R. H. *et al.* (1977) *supra.* for *G. arboreum* callus initiation. Including MS vitamins and/or 5% (v/v) coconut milk in some media such as EF18 were helpful in overall callus growth, but still these were inferior to G_2 medium.

SMpi medium (7.5 mg/l 2iP and 0.1 mg/l pCPA, Firoozabady, E. (1986), *supra* and SMgpi (SMpi with glucose instead of sucrose) were good for callus proliferation and maintenance, but still inferior to G2. Relatively hard, creamy granular calli were produced on media SMpi and SMgpi.

Rapid callus initiation on G_2 medium and proliferation on SMpi or SMgpi media indicating high level of 2iP or high 2iP/auxin ratio is important in these processes.

A range of gross morphology of the initiated calli was observed and varied from hard (nonfriable) to extremely friable, midfriable being the desired morphology. The degree of friability was highly dependent on the hormones used. For example, less friable tissues were obtained with higher levels of 2iP and higher 2iP/auxin ratios. Friability generally increased with higher NAA concentrations. Inclusion of 2,4-D in the media resulted in production of hard, compact calli. When no hormone was included in the subculture medium, midfriable tissues were obtained.

Embryogenic callus induction. Embryogenic calli were characterized as midfriable creamy granular tissues. Embryogenic calli were observed first as sections of calli or sometimes whole brown midfriable calli became embryogenic. These contained proembryoids (globular and heart-shaped structures). Transferring calli from EF18 to EFs18 (EF18 containing sucrose instead of glucose) was predicted by Shoemaker, R. C. *et al.* (1986) *supra.* to induce embryogenic callus formation. With Coker 201 and 315, this was possible at a very low frequency (2-3%, Table 15), but other cultivars did not respond to this exchange of carbohydrates. Calli on EFs18 (and other sucrose containing-media) generally produced a lot of phenolics, turned brown arid eventually died. With the subculture medium used by Davidonis, G.H., *et al.* (1983) *supra.*, only Coker 201 and 310 produced embryogenic calli (data not shown). In this work, media G1 and G3 containing 5 mg/l NAA produced massive amounts of embryogenic calli over several cultivars tested (Table 15), indicating that NAA is important for embryogenic callus induction. The fact that G3 was better than G1 also indicates that a high NAA/2iP ratio is beneficial for embryogenic callus induction in cotton.

Use of germinated immature seeds as the source of explants resulted in a higher rate of embryogenic callus formation in GSA lines but not in Coker 201 and 315.

Maturation and germination of somatic embryos and plant regeneration. After three to four weeks (Cokers) or approximately four months (GSA'S) on embryogenic callus induction medium (G_1 or G_3), embryogenic calli containing globular through torpedo stage somatic embryos were transferred to hormone-free (G_0) medium or sometimes to zeatin/NAA (MS_{zn-g}) containing medium. This subculture resulted in maximum embryogenic callus production (Table 15) and within two to three weeks, numerous tulip-shaped and mature embryos developed. Medium G_0 was best for somatic embryo maturation with several cultivars (Coker 201, 310, and 4360); GSA 78; and hybrid No. 21) tested. MS_{zn-g} was the second best in this regard. Replacing NAA with 2,4-D or pCPA in MS_{zn-g} media, greatly reduced their capacity for embryo production and maturation.

Embryos were very different in size (2-12 mm range) and morphology. They had different numbers of cotyledons (1-4), hypocotyl length, and colors (dark green, light green, and pale yellow).

Somatic embryos were transferred onto a lower ionic strength medium (GRM_{gn}) to germinate. GRM_{gn} was the best medium for embryo germination and plantlet development (Tables 16 and 17). Eliminating GA_3 and NAA reduced germination frequencies of the somatic embryos (GRM, Table 16). Generally, approximately 50% mature somatic embryos (normal or abnormal) germinated on GRM_{gn} (Tables 16 and 17). Larger embryos (8-12 mm, mature)germinated more frequently (approximately 90%) than small embryos (2-4 mm, immature). Usually G_1/G_3 - G_0 - GRM_{gn} resulted in higher frequencies of embryos germination and plantlet development than G_1 - GRM_{gn}, G_3 - GRM_{gn}, or any other transfer regimes (Table 17). This was probably due to higher frequencies of normal embryos developed on G_0 medium. Most germinated embryos produced both roots and leaves (plantlets). At the time of this writing in Coker 201, 255; Coker 310, 2; Coker 315, 6; Coker 4360, 7; No. 21, 4; No. 22, 3; GSA 71, 12; GSA 75, 20; GSA 78, 26 and GSC 30 plants have been regenerated and successfully transferred to soil and green house conditions.

Most of the regenerated plants have normal morphology, are fertile and have set seed.

Effects of light and temperature on different phases of cotton tissue culture. Different phases of cotton tissue culture were affected both by light and temperature, although these factors had less influence than medium composition. For callus initiation, a high temperature (30°C) was preferred and light intensity did not play an important role. Calli have been initiated and maintained at the same rate in complete darkness and in different light intensities (9-90 $\mu E/m^2/s$). For embryogenic callus formation and proliferation, high temperature and low light (9 $\mu E/m^2/s$) were preferred in the varieties tested: Coker 201 and 315 and GSA 78. After somatic embryos were formed, high light intensity (90 $\mu E/m^2/s$) proved to be very helpful for germination and plantlet development. It was also very helpful to incubate mature embryos at 30°C for a few days to rapidly germinate, then at 25°C for plantlet development. At high temperatures, the embryos and plantlets often grew slowly and callused.

Other observations on cotton regeneration. Embryogenic calli proliferated on G_6 medium (same as G_0 but with 6% glucose and 100 mg/l asparagine) with little mature embryo production, indicating the effects of high glucose and asparagine on embryogenic callus proliferation. Somatic embryos and plantlets in Coker 201 and 4360 have been induced to form callus (on G_2 medium) or embryogenic callus (on G_6 medium) and to undergo a second cycle of plant regeneration (Fig. 4) .

Inclusion of 5 rpm ancymidol or 100 mg/l asparagine in GRM medium resulted in healthier plantlets; the plantlets had dark green leaves and a better root system.

Some somatic embryos and plantlets developed abnormally. For example, occasionally, embryos were formed on previously matured embryos, some plantlets produced callus on top or on the stem, and some produced many slim shoots. However, these abnormal tissues could be induced to form callus and subsequently regenerate normally.

Some calli from GSA 78 and GSA-Acala hybrid No 21 have spontaneously regenerated shoots via organogenesis. However, to date we have only been able to regenerate plants from these shoots at frequencies of approximately 25% because of difficulties in inducing root formation.

Summary of results. A highly efficient and general method for plant regeneration in cotton, *G. hirsutum* has been developed. The method is very rapid for Class 1 cultivars (Cokers --14-18 weeks) and relatively slow for Class 2 cultivars (GSA's -- 8-10 months). For Class 2 cultivars developmental stages progressed more slowly than for Class 1 cultivars, and with lower efficiency of regeneration. Use of precociously germinated seedlings as the source of explants increased frequencies of embryogenic calli formation with Class 2 but not with Class 1 cultivars. Embryogenic cultures are very stable, and upon monthly subculture on G_0 medium, numerous somatic embryos are produced; at this writing plants are still being regenerated from 15-month-old Coker 201 and GSA 78 embryogenic callus lines.

In comparing different media formulations, it is clear that a high 2iP/auxin ratio is best for callus initiation and proliferation but must be exchanged for a high NAA/cytokinin ratio before embryogenic calli will form. Interestingly, in *G. hirsutum* the ability to form embryogenic calli appears to be influenced by NAA (this study, Davidonis, G. H., *et al.*, *supra* Shoemaker, R. C. *et al.*, *supra*). We have found that continuous subculture on high levels of 2iP suppresses morphogenesis. However, upon two subcultures each on induction medium (G_1 or G_3 and embryo formation medium (G_0) embryos are formed.

For maximum somatic embryo formation, it was essential to subculture calli hormone-free medium. The length of time on hormone-containing medium (i.e., G_1 or G_3), however, had an effect on embryo formation.

In the present study, since over 100 embryos were formed per dish, an ample number was readily obtained to test the effects of various cultural conditions on embryo germination and plant development. Germination of somatic embryos was highly dependent on frequency of normal embryos and ionic strength of the medium. Abnormal embryos rarely germinated under any

condition and had a long lag period. Small embryos also germinated infrequently and slowly. Normal and large embryos (greater than 5 mm), however, germinated well on appropriate media. Embryos placed on BT medium (Beasley, C. A., *et al.*, *supra.*) either developed callus or their growth stopped and all tissues became necrotic. Embryos were unable to tolerate high- salt (Table 16, EG) or high-sugar (Table 16, EG, EG_1) media, and *myo*-inositol was partially inhibitory to root development (Table 16, EG1, EG3). Often root or shoot development was a problem, and callusing was very common. The embryos performed best on modifications of a medium recommended by Stewart, J. N., *et al.*, *supra.*, GRM_{gn}. Addition of compounds such as asparagine and ancymidol to GRM_{gn} proved helpful in embryo development. Balanced root and shoot growth was obtained using the medium GRM_{gn}. Embryo growth was slower in the medium lacking hormones (GRM). Embryo germination frequencies were higher on GRM_{gn} medium in cases where embryos matured on hormone-free medium, G_0, due to production of higher frequencies of normal embryos on G_0 medium (Table 17). On hormone-containing media, G_1 and G_3, proembryoids grew slowly and many callused and proliferated instead of undergoing maturation.

COTTON TRANSFORMATION EXAMPLE

The neomycinphosphotransferase II (*npt* II) gene confers kanamycin resistance in eukaryotes when inserted behind eukaryotic promoters. The *npt* II coding sequence from bacterial transposon Tn5 was inserted behind the cauliflower mosaic virus (CaMV) 19S promoter and was terminated by addition of T-DNA 0RF26. This chimeric gene, T-DNA OCS gene, *npt* II gene, and A and B borders of T-DNA was inserted into the broad host range replicon pTJS75 to create pH575. The construction of this vector is more fully described in U.S. Patent Applications No. 788,984 filed October 21, 1985, and incorporated herein by reference.

Agrobacterium tumefaciens strain LBA4404 carrying pH575, and LBA4404 without the vector were cultured on Luria broth agar medium containing 250 µg/ml streptomycin and 25 µg/ml kanamycin (both from Sigma) for selection. Strain LBA4404 is described in Hoekema, *et al.* (1983) Nature *303*:179. This is a widely available strain also freely available from the authors. Bacteria were scraped off the agar medium, suspended in a liquid medium for cotton callus culture (G_2) to a concentration of 2-4 X 10^8 cells/ml and were used for inoculation of cotyledon segments.

To transform cotton tissues, cotyledon pieces (approximately 0.5 cm^2 surface area) from sterile 12- to 14-day- old Coker 201 seedlings were dipped in *A. tumefaciens* liquid cultures in Petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial

cultures. The cotyledon pieces were then blotted dry and plated on Whatman #1 filter paper on a callus initiation medium (G_2)containing MS salts (Gibco), 100 mg/l *myo*-inositol, 0.4 mg/l thiamine HCL, 5 mg/l 2iP, 0.1 mg/l NAA (all from Sigma), 3% (w/v) glucose, 0.2% Gel-rite (Kelco), pH5.9. Filter paper was not necessary for transformation, but greatly reduced bacterial overgrowth on plant tissues. After three days cultivation at low temperature (25°C) and low light, cotyledon pieces were transferred to Petri plates containing the same medium, without the filter paper, and containing 500 mg/l carbenicillin and 25 mg/l kanamycin sulphate (both from U.S. Biochemicals).

After 7-10 days of incubation, cotyledon pieces initiated transformed kanamycin-resistant microcalli (0.5 mm) at wound sites, while no callus from control untreated tissues or from tissues treated with LBA4404 grew on kanamycin. Two to three weeks later, 2-4 mm calli were excised from original explants and transferred to fresh medium. All the calli were kanamycin-resistant and 80-100% of the kanamycin-resistant calli were positive when tested for octopine (Table 18). Two to three weeks later, the calli were placed and maintained on embryogenic medium G_O (same as G_2 with no hormones) under selection. Mature somatic embryos were transferred to lower ionic strength medium, GRM_{gn} described above. Plants were shown to be transformed by their resistance to kanamycin in leaf callus assay, production of octopine, enzyme-linked immunoassay (ELISA) for NPT II (Table 19) and by DNA, and western blot hybridization analyses. Plants were transferred to soil for further analysis. The whole process from infection until transgenic plants were transferred to soil took about six months. Similar results were obtained in replicate experiments.

Transgenic cotton plants are readily obtained with the transformation-regeneration system described here. The system is very efficient and has been modified to be applicable for plant regeneration of a number of commercial cultivars of cotton, although the efficiency and time period for regeneration of other cultivars are not as short as for Coker 201. The use of a selectable kanamycin-resistance marker was necessary. Kanamycin-resistant microcalli developed at the wound sites. In the absence of selection, barely detectable octopine-positive calli were obtained in frequencies of 10-20% indicating transformation frequencies were reasonably high among the cell populations of some calli.

Generally, 150-200 calli per 100 cotyledon sections were produced on kanamycin; 100% of these survived during subculture and remained to be resistant to kanamycin; about 80-100% of these were octopine-positive and in Coker 201 more than 80% became embryogenic and regenerated into plants. At this writing, approximately 86 octopine-positive plantlets have been produced using different constructs. About 30% of these regenerated into normal plants. Six of the octopine-positive

plants tested showed the DNA fragments of appropriate size showing *ocs*, *NPT* II, and *bt* genes. In another experiment eight octopine-positive plants and 10 octopine-positive calli tested were positive for NPT II protein in western blot and in ELISA. All octopine-positive plantlets so far tested were kanamycin-resistant in leaf callus assay. The efficiency of this transformation-regeneration system permits the introduction of desirable genes such as insect-resistance, herbicide-resistance and virus-resistance to cotton. Cotyledon pieces (approximately 0.5 cm^2 surface area) from sterile 12 to 14 day old coker 201 seedlings were dipped in *A. tumefaciens* liquid cultures in Petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial cultures. the cotyledon pieces were then blotted dry and plated on Whatman #1 filter paper on a callus initiation G_2 medium containing 0.2% "GEL-RITE" (Kelco). After three days cocultivation at low temperature (25°C) and 16 h/day photoperiod at 90 $\mu E \cdot m^{-1} \cdot s^{-1}$ light, cotyledon pieces were transferred to Petri plates without the filter paper containing the same medium supplanted with 500 mg/l carbenicillin and 25 mg/l kanamycin sulfate (both from U.S. Biochemicals).

After page 123, after Table 13, please insert the following Tables 14-19:

Table 14: Media Composition Used for Cotton Regeneration[1]

Medium	NAA (mg/l)	2iP (mg/l)	IAA (mg/l)	kinetin (mg/l)	zeatin (mg/l)	MS salts	% (w/v) glucose (g) or sucrose (s)
$1/2G_0$						1/2X	1.5g
G_0						1X	3.0g
G_1	5	1				1X	3.0g
G_2	0.1	5				1X	3.0g
G_3	5	0.1				1X	3.0g
G_4			0.1	1		1X	3.0g
G_5			2	1		1X	3.0g
EF_18	2			1		1X	3.0g
MS_{zn-g}	0.1				1	1X	1.5g
EG_1				1		1/2X	1.5s
EG_3	0.01					1/2X	0.5g

[1]All media were prepared with MS salts (Gibco, Grand Island, NY), 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 0.2% Gel-rite (Kelco, San Diego, CA), pH 5.9. The remaining media used have already been published or are mentioned in the text. All chemicals (unless indicated) were purchased from Sigma Co. (St. Louis, MO). IAA and zeatin were filter sterilized, and the rest were autoclaved.

Table 15: Effects of different media on cotton embryogenic callus frequency of Cokers 10 weeks and GSA's 8 months after explantation. A total of 30-40 calli per cultivar were tested.

Initiation & induction media	Embryogenic calli formation medium	% Embryogenic calli[1]				(% Embryogenic mass)[2]			
		Coker				GSA			
		201	208	310	315	25	71	75	78
EF18	EFs18	3(1)	0	0	2(1)	0	--	--	0
$G_2 - G_3$	½ G_0	--	--	--	--	0	3(1)	2(1)	--
$G_2 - G_3$	G_0	85(75)	49(38)	61(52)	80(68)	(4)	10(4)	12(4)	20(15)
$G_2 - G_1$	G_1	50(41)	(17)	(18)	(12)	(3)	0	--	--
$G_2 - G_3$	G_3	54(45)	(31)	(32)	(15)	(3)	(6)	(4)	--
$G_2 - G_4$	G_4	(26)	(16)	(1)	(8)	--	--	--	--
$G_2 - G_5$	G_5	(14)	--	(1)	(10)	--	--	--	--

[1]Based on numbers of calli.

[2]% Embryogenic mass was estimated as $\frac{\text{mass of embryogenic calli}}{\text{total mass of calli}}$ X 100

Table 16: Effects of different media on cotton somatic embryo[1] germination and plantlet formation. (Total number embryos are shown with percentage of embryos germinated and formed plantlets, respectively.)

Medium	Coker 201	Coker 310	Coker 315	Coker 4360	GSA78
GRM_{gn}	650 (75, 55)	30 (80, 20)	41 (46, 24)	28 (36, 25)	152 (72, 56)
EG_1	83 (30, 16)	24 (67, 17)	23 (52, 4)	26 (38, 15)	--
EG_3	175 (48, 25)	25 (68, 4)	16 (50, 6)	44 (41, 2)	--
GRM	71 (51, 30)	--	--	--	--
EG^2	43 (47, 12)	--	--	--	--

[1]Somatic embryos were selected randomly so they consisted of normal, abnormal, small and large embryos, and they were distributed randomly over different media.

[2]Embryo germination medium used by Shoemaker, R.C. *et al.*, *supra.* containing MS salts, 1 mg/l kinetin and 1.5% sucrose.

Table 17: Effects of embryo formation and embryo germination media on Coker 201 embryo germination and plantlet development.[1]

Induction medium	Embryo Formation Medium	% Normal Embryos	Embryo Germination Medium	Total Number Embryos Plated (% Germinated, % Formed Plantlets)
G_1, G_3	G_0	44	G_0	25 (24, 24)
			G_1	16 (44, 33)
			EG_3	25 (40, 32)
			GRM_{gn}	46 (63, 54)
G_1	G_1	14	G_1	23 (22, 13)
			GRM_{gn}	30 (27, 23)
G_3	G_3	32	G_0	26 (38, 19)
			G_1	16 (19, 17)
			EG_3	35 (31, 23)
			GRM_{gn}	35 (37, 37)

[1]Calli were initiated from hypocotyl sections on G_2, after three weeks transferred to G_1 or G_3, and after another three weeks were transferred to embryo formation media.

Table 18: Transformation Frequencies of Cotton Cotyledon Segments.

		Transformation Frequencies (%)			
		Growing callus in the presence of kanamycin at (μg/ml)			Octopine Positive
Construct	Cultivar	25	50	100	
pH575	Coker 201	75	78	85	33
	GSA 75	40	54	43	32
LBA4404	Coker 201	20	13	2	0
	GSA 75	14	11	0	0

Table 19.

Sample	NPTII ng/ml	protein g/ml	ngNPT/mg protein % expression
1	325.9	8926	36.6
2	209.7	11744	17.9
3	714.7	14336	49.9
4	238.2	14673	16.2
5	318.4	10470	30.4
6	349.4	9489	36.8
7	253.5	9151	27.6
8	144.1	14335	13.4
9 (neg. cntrl)	0.0	10770	0.0

After Figure 3, Please insert the attached Figure 4.